Biochemical Pharmacology, Vol. 21, pp. 2373–2383. Pergamon Press, 1972. Printed in Great Britain.

DIETHYLAMINOETHYL 2,2-DIPHENYLVALERATE HCl (SKF 525-A)—*IN VITRO* AND *IN VITRO* EFFECTS OF METABOLISM BY RAT LIVER MICROSOMES—FORMATION OF AN OXYGENATED COMPLEX*

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(*Received* 27 *December* 1971; *accepted* 10 *March* 1972)

Abstract—Metabolism of diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) or its primary amine analogue causes formation of a stable oxygenated complex of ferrous P-450. The complex is also generated *in vivo* and survives preparation of liver microsomes. Formation of the complex requires active metabolism of either SKF 525-A or the *N*-dealkylated primary amine, SKF 26754A. This new species has an absorption maximum at 455 nm. Oxidation of the hemoprotein with potassium ferricyanide causes loss of the absorption band, but subsequent reduction and oxygenation of the medium restore it. This new complex is believed to be responsible for the observed noncompetitive inhibition of drug metabolism *in vitro* by SKF 525-A.

IN THE early 1950's, a number of reports appeared indicating that a new compound, diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A), potentiated the pharmacological actions of analgesic drugs such as methadone and morphine,[1] spinal cord and central nervous system depressants as 2-amino-6-methylbenzothiozole and mephenesin, some central nervous system stimulants such as amphetamine and the convulsant effects of strychnine,[2] as well as the effects of hypnotic drugs.[3] These effects were reported to be the result of a marked diminution in the rates of biotransformation of the drugs.[4,5]

Since these early studies, SKF 525-A has been shown to inhibit the metabolism of most substrates of the hepatic microsomal mixed function oxidase system,[6] as well as phenolic glucuronidation[7] and nitro reductase activity *in vitro*.[8] Inhibition of the microsomal drug oxidase by SKF 525-A was suggested by Brodie[9] as being caused by interaction of the inhibitor with the cellular membranes, thereby blocking in some way the penetration of drug substrates to the enzyme. Netter[6] proposed that the inhibition was due to an uncoupling of the microsomal electron transport system. Gillette and Sasame,[10] however, observed that irreversible inhibition of drug metabolism occurs when microsomes are preincubated *in vitro* with NADPH and SKF 525-A; this effect was not obtained in the absence of NADPH or air, nor could it be removed by extraction with boiled liver microsomes. Rogers and Fouts[11] found SKF 525-A to be strongly bound to liver microsomes, but to be almost absent 12 hr after administration *in vivo*; yet at this time the amount of enzyme inhibition remained essentially unchanged, the metabolizing activity recovering to above normal levels after 48 hr.

* Supported in part by a National Science Foundation Grant, GB18660 and by National Institutes of Health Grants GM17021 and CA10748.

† Career development awardee of the National Institutes of Health, GM-K4-19, 601.

The study reported in this paper was undertaken to learn the nature of the observed inhibition of drug oxidation by SKF 525-A and how this inhibition develops.

MATERIALS AND METHODS

In studies *in vivo*, male and female Sprague–Dawley rats (50–60 g) were injected intraperitoneally twice daily with 25 or 50 mg/kg of SKF 525-A for 7 days to 3 weeks. The SKF 525-A was dissolved first in distilled water, precipitated by the addition of 1 N NaOH (to pH about 7–8) and was then extracted into corn oil (Mazola) for injection. Control rats were injected with corn oil alone.

Liver microsomes were prepared as described previously.[12] Aminopyrine demethylation was determined from formaldehyde production, and aniline hydroxylation was determined by *p*-aminophenol formation as described previously.[13] Off-balance absolute spectra were obtained as shown by Remmer *et al.*[14] Lineweaver–Burk kinetic plots were determined by visual means and did not differ from the curves obtained by the method of least squares.

Aminopyrine was obtained from Matheson, Coleman & Bell, N.J.; SKF 525-A and SKF 26754A were the generous gifts of Smith, Kline & French Laboratories, Philadelphia, Pa. Aniline was obtained from Fisher Co. and was redistilled under vacuum before use.

RESULTS

When SKF 525-A was added to the assay medium with another substrate of the mixed function oxidase, but prior to the addition of microsomes, it too behaved as a substrate of the enzyme system, competitively inhibiting the metabolism of the known substrate (Fig. 1); both aminopyrine *N*-demethylation (Fig. 1A) and aniline hydroxylation (Fig. 1B) were competitively inhibited by the presence of 0·05 and 0·1 mM SKF 525-A. However, when the microsomes were preincubated with SKF 525-A in the presence of NADPH (generating system + NADP) for 5 min prior to the addition of another substrate, the observed inhibition was noncompetitive (Fig. 2); more NADP was added with substrate to assure that sufficient NADPH was present. In Fig. 2, A and B are shown the noncompetitive inhibition of aniline hydroxylation and aminopyrine *N*-demethylation by 0·05 and 0·1 mM SKF 525-A; the V_{max} activities after preincubation were 5 and 20 per cent lower, respectively, in the absence of the inhibitor.

In order to determine the nature of these effects, we turned to spectral examination of the microsomes in the presence of SKF 525-A and N-diethyl compound and its primary amine homologue, SKF 26754A. On addition of SKF 525-A to the microsomal suspension in the absence of a source of reducing equivalents, a type I spectral change is obtained.[12,13] The addition of NADPH to the sample cuvette caused an absorption peak to develop with time (Fig. 3) at 455 nm. The development of the 455 nm absorption peak was slow, requiring about 15 min before it was pronounced; it was about maximal by 1 hr. Oxygen was bubbled into the cuvettes for 1 min at 10-min intervals to prevent its depletion and 1 mM NADPH was added at 15-min intervals. In Fig. 3, curve a is seen at 1 hr after NADPH addition. The NADPH was exhausted in the sample cuvette, but some still remained in the reference cuvette; this caused a shift in the peak due to the presence of some reduced cytochrome b_5

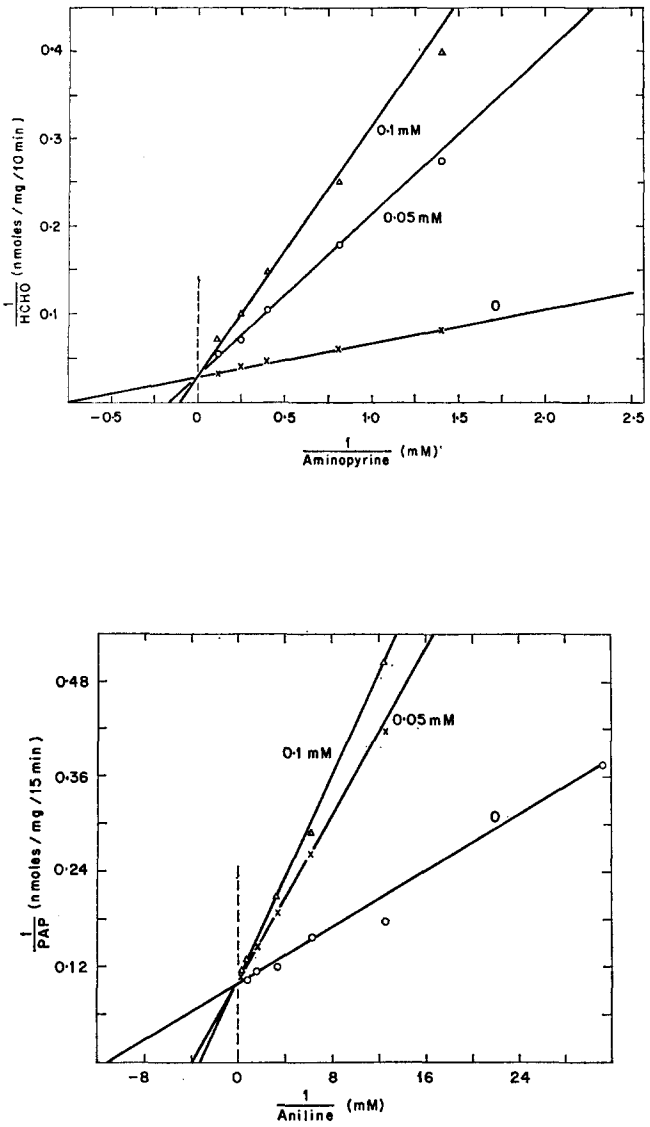
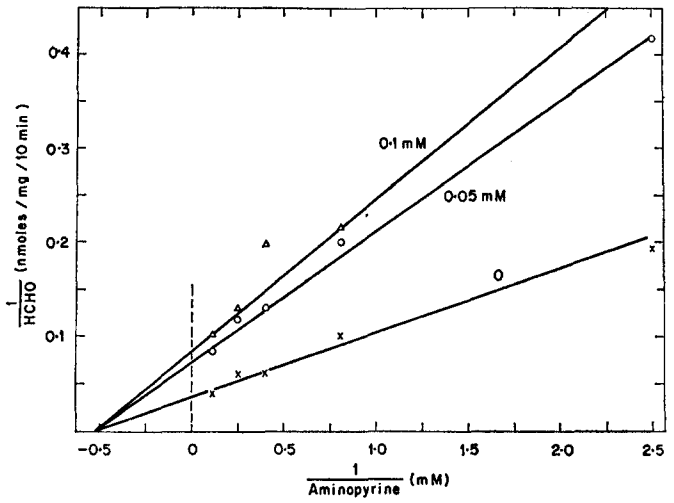


FIG. 1. Effects of SKF 525-A addition on mixed function oxidations. (A) Aminopyrine demethylation. Lineweaver–Burk plot of enzyme activity in the presence of 0·05 mM, 0·1 mM or no SKF 525-A. The inhibitor and substrate were present in the assay medium which was preincubated for 5 min at 37° to generate NADPH (from 0·35 mM NADP). The microsomes (1 mg/ml) were added to the medium and incubation was continued for 10 min at 37°. Activity is expressed as nanomoles HCHO per 10 min per milligram of microsomal protein. The K_m (mM) and V_{max} (nanomoles per milligram per 10 min) in the presence of 0, 0·05 and 0·1 mM SKF 525-A were 1·25 and 40, 7·10 and 40 and 8·33 and 40, respectively, by the method of least squares. (B) Aniline hydroxylation. Conditions were as in A, with aniline replacing aminopyrine. Activity is expressed as nanomoles of *p*-amino–phenol per 15 min per milligram of microsomal protein. The K_m (mM) and V_{max} (nanomoles per milligram per 15 min) in the presence of 0, 0·05 and 0·1 mM SKF 525-A were 0·09 and 10, 0·25 and 10 and 0·30 and 10, respectively, by the method of least squares.



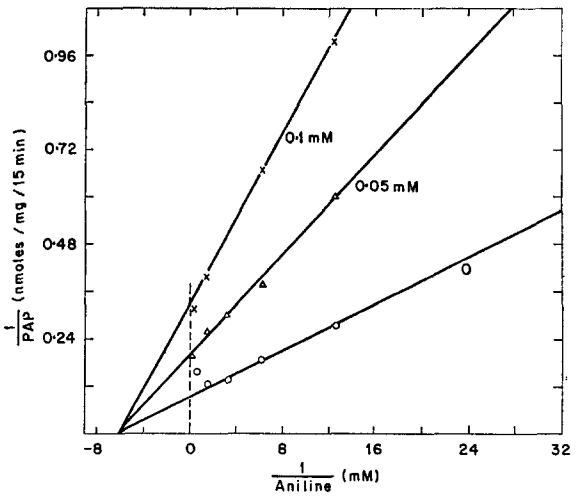


FIG. 2. Effect of microsomal metabolism of SKF 525-A on the biotransformation of other drugs. (A) Aminopyrine demethylation. Lineweaver–Burk plot of enzyme activity after preincubation of functioning assay system with 0·05 mM, 0·1 mM or no SKF 525-A for 5 min before addition of different concentrations of aminopyrine. More NADP (an additional 0·35 mM) was added with aminopyrine. Microsomal content was 1 mg/ml. The K_m (mM) and V_{max} (nanomoles per 10 min) in the presence of 0, 0·05 and 0·10 mM SKF 525-A were 1·9 and 28, 1·9 and 14 and 1·9 and 12, respectively, by the method of least squares. (B) Aniline hydroxylation. Conditions were as in A, with aniline replacing aminopyrine. The K_m (mM) and V_{max} (nanomoles per milligram per 15 min) in the presence of 0, 0·05 and 0·10 mM SKF 525-A were 0·16 and 11, 0·16 and 5 and 0·16 and 3, respectively, by the method of least squares.

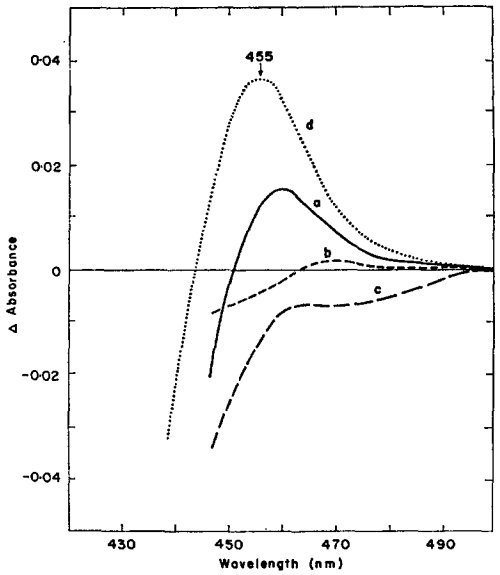


FIG. 3. Generation of the 455 nm peak during SKF 525-A metabolism. Microsomes were suspended to 2 mg protein/ml in 0·1 M Tris, pH 7·5, and the suspension was distributed between two cuvettes. SKF 525-A, 0·1 mM, was put into one cuvette and 1 mM NADPH was added to both cuvettes at 15-min intervals, followed by gassing for 1 min with oxygen. Curve a, difference spectrum at 1 hr; curve b, 20 μl of 1 mM potassium ferricyanide added to both cuvettes; curve c, addition of sodium dithionite (about 0·5 mg) to each cuvette; curve d, after gassing each cuvette for 5 min with O_2.

in the reference cuvette. The addition of potassium ferricyanide (curve b) as a chemical oxidant caused the disappearance of the 455 nm band. This absorption peak was not due to the presence of carbon monoxide, because addition of the chemical reductant dithionite to each cuvette (curve c, Fig. 3) did not restore the 455 nm peak. When oxygen was bubbled into each cuvette for 5 min to remove the dithionite, the 455 nm peak was restored to its full height, indicating that it is an oxygenated form of cytochrome P-450 or of a cytochrome P-450 metabolite complex.

Since SKF 525-A is metabolized by *N*-dealkylation, it seemed possible that in our system we were generating the secondary amine, SKF 8742A, or the primary amine, SKF 26754A, which then formed a ferrihemochrome. Anders *et al.*[15] reported that metabolism of SKF 525-A yielded the monoethyl compound, but the monoethyl compound (SKF 8742) caused a small type I spectral change (B. Wilson and J. B. Schenkman, unpublished observations) when added to liver microsomes. When the primary amine was added to a microsomal suspension (Fig. 4), it caused the appearance of a type II spectral change with a peak at 432 nm (curve a) and a trough at 700 nm. The addition of NADPH to each cuvette, however, resulted in the generation with time of the same 455 nm peak as with SKF 525-A (curves b, c and d). The only difference between the two preparations was that the 455 nm peak developed more rapidly with the primary amine (about $\frac{1}{4}$ of the time). As observed during metabolism of SKF 525-A, the peak disappears when either potassium ferricyanide (curve e) or dithionite is added; bubbling oxygen into the microsomal suspension after dithionite restores the 455 nm peak.

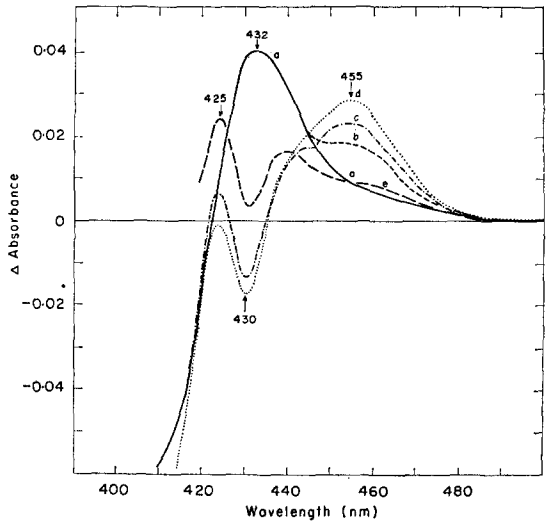


FIG. 4. Generation of the 455 nm peak during SKF 26754A metabolism. Microsomes were suspended to 2 mg protein/ml in 0·1 M Tris, pH 7·5, and the suspension was distributed between two cuvettes. SKF 26754A, 0·18 mM, was added to one cuvette and a spectrum was recorded (curve a). Then 1 mM NADPH was added to each cuvette and spectra were recorded after 10, 20 and 30 min (curves b, c and d); more NADPH was added 15 min after the first addition of the pyridine nucleotide. The effect of addition of 1 mM $K_3Fe(CN)_6$ to each cuvette is shown in spectrum e.

The possibility that this absorption peak was merely an oxygenated form of cytochrome P-450 was obviated by adding dithionite to one cuvette containing microsomal suspension (without SKF 525-A) and then bubbling oxygen to remove the dithionite; no 455 nm peak appeared. Furthermore, other substrates of the mixed function oxidase, like aminopyrine and hexobarbital, did not cause the appearance of a peak in the absorption spectrum, suggesting that some metabolite of SKF 525-A (or SKF 26754A) was bound to cytochrome P-450, and stabilized the hemoprotein in the reduced oxygen-bound form.

This possibility was tested by administration of SKF 525-A *in vivo*, on the assumptions that such a metabolite would not readily be removed from the hemoprotein and that the 455 nm compound should be readily seen in isolated microsomes. That these assumptions were correct is shown in Figs. 5 and 6.

When SKF 525-A was administered twice daily at a dose of 25 mg/kg to female rats for 3 weeks, it acted as an inducer of the hepatic microsomal mixed function oxidase system but not of cytochrome P-450. Analyses of drug oxidase activity indicated an increase in aminopyrine and ethylmorphine demethylation activities and aniline hydroxylation activity (2·5-, 2·8- and 1·2-fold respectively) per milligram of microsomal protein; no changes in Michaelis constants (K_m) were observed.

Examination of the microsomes by off-balance spectroscopy[14] revealed differences in the absorptive properties (Fig. 5A). Although cytochrome P-450 levels were almost the same in control and treated animals, the hemoproteins were not in the same form. Some of the cytochrome P-450 of the treated rats was present in the form (enzyme–substrate complex) absorbing further in the ultraviolet region (peak 384 nm) and some was present in the form absorbing at 454 nm. In Fig. 5A, the trough appearing

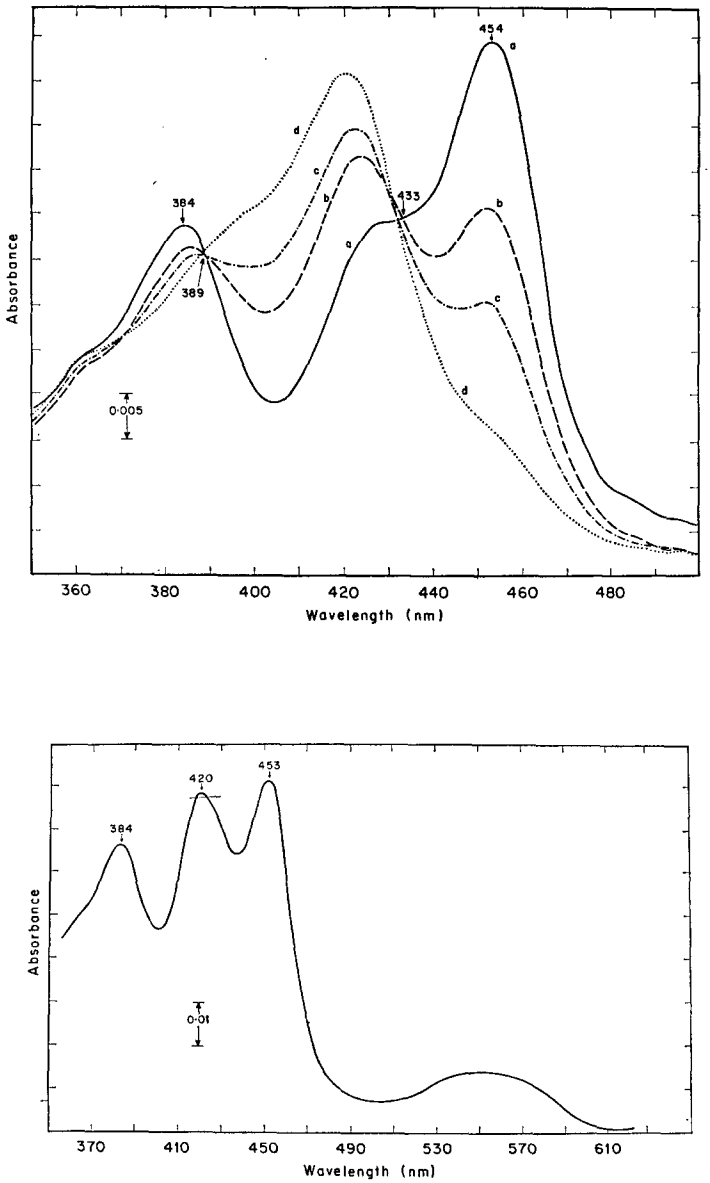


FIG. 5. Off-balance absolute spectrum of liver microsomes of rats treated with SKF 525-A. (A) Sample cuvette contained 1·5 mg protein/ml from treated female rats; hemoprotein content was 0·85 mM cytochrome b_5 and 1·29 mM cytochrome P-450. Reference cuvette contained 1·5 mg microsomal protein/ml from untreated female rats; hemoprotein content was 0·85 mM cytochrome b_5 and 1·21 mM cytochrome P-450. Curve a shows the off-balance absolute spectrum of the liver microsomes, the difference between treated and untreated female rats. The effects of consecutive additions of 0·5 mM concentrations of $K_3Fe(CN)_6$ are shown in spectra b, c and d. Weanling female rats were given 25 mg SKF 525-A/kg twice daily for 21 days. (B) Sample cuvette contained 2·0 mg liver microsomal protein/ml from treated male rats; hemoprotein content was 0·88 mM cytochrome b_5 and 1·88 mM cytochrome P-450. Reference cuvette contained 2·0 mg liver microsomal protein/ml from untreated male rats; hemoprotein content was 0·88 mM cytochrome b_5 and 1·26 mM cytochrome P-450. Weanling male rats received 25 mg SKF 525-A/kg twice daily for 8 days.

maximally at 410 nm is probably due to more cytochrome P-450 present in the usual 420 nm-absorbing form (non-substrate bound) in the control rat liver microsomes. The absorption peak at 454 nm is diminished by the addition of $K_3Fe(CN)_6$ solution (Fig. 5A) with the concomitant appearance of the absorption peak at 420 nm. There is a slight decrease in absorption at 384 nm too, with a resultant appearance of two isosbestic points, one at 389 and the other at 433 nm. If one assumes an extinction coefficient of about 100 mM^{-1} cm^{-1} for the 420 nm peak, the increase in 420 nm-absorbing hemoprotein is about the amount of excess P-450 in the sample cuvette. However, as indicated earlier,[16] because of differences in background absorbance and turbidity, calculation of extinction coefficients for the different forms by off-balance spectroscopy is not possible.

Similar observations were obtained with male rats. After 7 days of twice daily injections of 25 mg SKF 525-A/kg of body weight, only a slight increase in cytochrome P-450 was observed (Fig. 5B). However, off-balance spectroscopy revealed absorption peaks at 384, 420 and 453 nm in the near ultraviolet-Soret region; in the visible region, a broad absorption band appeared between 540 and 570 nm. Again, although only a slight difference in hemoprotein P-450 existed, SKF 525-A pretreatment caused a 2-fold higher aminopyrine demethylase activity (V_{max}) and a 30 per cent higher aniline hydroxylase activity (V_{max}), both without alteration in K_m values.

Because the possibility existed that due to a rapid metabolism of the SKF 525-A *in vivo* we were not seeing maximal effects of SKF 525-A, the daily injections of the drug were doubled to two doses of 50 mg/kg. At this level SKF 525-A behaved like a potent inducer of cytochrome P-450, increasing its content in the microsomes about 2·5-fold. When these microsomes were examined by off-balance spectrophotometry (Fig. 6), the peaks at 385 and 454 nm again appeared, along with the usual cytochrome

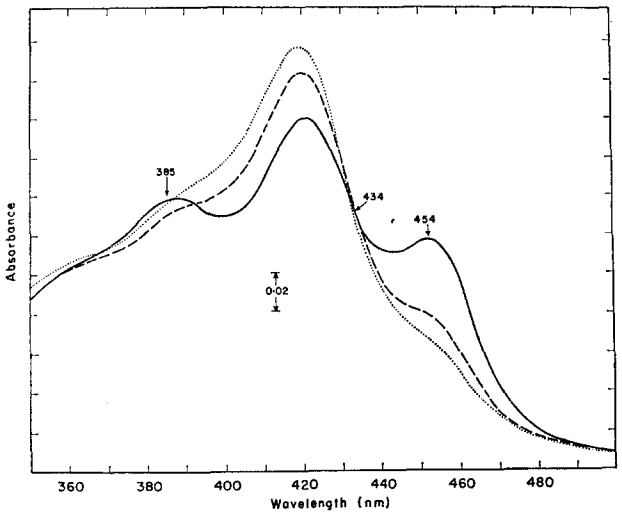


FIG. 6. Off-balance spectrum of microsomes from SKF 525-A-treated female rats. Rats were treated twice daily with 50 mg SKF 525-A/kg for 1 week. Microsomal protein in sample (treated animal) cuvette was 1·2 mg/ml and contained 0·8 mM cytochrome b_5 and 1·96 mM cytochrome P-450. Reference cuvette contained 1·0 mg microsomal protein/ml from untreated animals; the b_5 content was 0·8 mM and P-450 content was 0·77 mM. Solid line, off-balance absolute spectrum; dashed line, 1 mM $K_3Fe(CN)_6$ added to each cuvette; dotted line, 2 mM $K_3Fe(CN)_6$ in each cuvette.

P-450 peak at 420 nm. Again, as in previous experiments, the addition of potassium ferricyanide caused the disappearance of the 454 nm peak with a concomitant increase in absorption at 420 nm. Addition of dithionite to these microsomes followed by gassing with oxygen did not restore the absorption peak, as it does during metabolism *in vitro* (Fig. 3). Concomitant with the increase in cytochrome P-450, there was a doubling of the aminopyrine demethylase activity (6·6 nmoles/min/mg to 12·4 nmoles V_{max}) without an alteration of K_m.

Although up to 100 mg SKF 525-A was administered/kg daily for 1–3 weeks, no ill effects were seen. There was normal body weight and organ weight gain. However, testicular weight was statistically greater in rats treated from weanling age (55 g) for 28 days with 50 mg/kg of the inhibitor daily (Table 1).

TABLE 1. EFFECT OF 28 DAYS OF SKF 525-A* TREATMENT ON BODY AND ORGAN GROWTH OF WEANLING MALE AND FEMALE RATS

Treatment	N†	Body wt. gain (g ± S.E.)	Liver wt./ body wt. × 100 (% ± S.E.)	Kidney wt./ body wt. × 100 (% ± S.E.)	Spleen wt./ body wt. × 100 (% ± S.E.)	Testes wt./ body wt. × 100 (% ± S.E.)
Control	9	97·7 ± 5·58	4·88 ± 0·12	0·84 ± 0·07	0·34 ± 0·02	
SKF 525-A	10	95·1 ± 2·93	5·14 ± 0·05	0·73 ± 0·02	0·34 ± 0·05	
% of Control		97	105	87	99	
Control	10	150·4 ± 7·18	4·91 ± 0·15	1·04 ± 0·06	0·51 ± 0·06	1·51 ± 0·05
SKF 525-A	8	125·7 ± 10·60‡	4·92 ± 0·08	1·09 ± 0·08	0·62 ± 0·08	1·78 ± 0·09§
% of Control		83	100	105	122	118

* SKF 525-A was extracted into corn oil and 50 mg/kg/day injected intraperitoneally (two injections/day).

† N represents the number of animals.

‡ Not statistically different.

§ $P < 0·05$.

DISCUSSION

The effects *in vitro* of addition of SKF 525-A to the medium depends upon whether prior metabolism of the inhibitor has occurred. In agreement with the report by Gillette and Sasame,[10] simultaneous addition of SKF 525-A and a drug to the medium caused a competitive inhibition of the latter metabolism (Fig. 1, A and B). This would be expected, since it has been shown[15] that SKF 525-A is itself a substrate for microsomal *N*-dealkylation activity. However, unlike the reports of Anders and Mannering,[17] Ikeda *et al.*,[18] and Kato *et al.*,[19] we have found inhibition of metabolism *in vitro* of different drug substrates to depend upon the extent of metabolism of the SKF 525-A; inhibition is competitive when both substrates are simultaneously introduced, but becomes noncompetitive when inhibitor metabolism has occurred. It is probably due to the long (30 min) assay period used that Ikeda *et al.*[18] obtained noncompetitive inhibition of aniline hydroxylation by SKF 525-A with guinea pig microsomes, when both drugs were introduced together.

In an attempt to discern the nature of this noncompetitive inhibition, liver microsomes were investigated spectrophotometrically during metabolism of SKF 525-A. It was found that during metabolism of SKF 525-A a stable oxygenated cytochrome

P-450 metabolite complex slowly developed. After generation of the 455 nm complex (determined spectrophotometrically), metabolism of aminopyrine was found to be inhibited noncompetitively (B. J. Wilson and J. B. Schenkman, unpublished observations). Two other substrates of the mixed function oxidase, aminopyrine and ethylmorphine, when incubated with liver microsomes and NADPH, did not generate the 455 nm peak, despite the fact that both are also metabolized by *N*-dealkylation. This would tend to rule out the possibility that the 455 nm form of the hemoproteins is just the oxygenated intermediate formed during metabolism of drugs as suggested by Estabrook *et al.*[20] In this vein, it is of interest that in a report by Ishimura *et al.*[21] in which a trapped oxygenated intermediate of cytochrome P-450 ($P\text{-}450_{cam}$) was described, the complex was shown to have a 418 nm peak. Recently, Estabrook *et al.*[22] also reported the formation of a new spectral species when hexobarbital was added to microsomes in the presence of NADPH; their new species had a broad band at 440 nm. However, under the experimental conditions used in our report, hexobarbital, aminopyrine, ethylmorphine and aniline all failed to generate a peak. This suggests that the peak seen by these investigators was a reduced form of cytochrome P-450 in complex with endogenously generated carbon monoxide, or a reduced minus oxidized difference spectrum of cytochrome P-450, like that observed by Omura and Sato.[23]

It was postulated that if the oxygenated form of cytochrome P-450 generated during metabolism of SKF 525-A and SKF 26754A were the result of stabilization of the hemoprotein by a metabolite, it should be observable after administration of SKF 525-A *in vivo*. When rats were treated with SKF 525-A twice daily at 50–100 mg/kg/day for periods of 7 days to 3 weeks, the isolated liver microsomes were found to contain the peak at 455 nm. No such peak was observed after comparable injections of phenobarbital or 3-methylcholanthrene,[16] indicating that its presence and survival of the preparative methods were probably due to stabilization by some metabolite of SKF 525-A. The 455 nm complex appeared even when no apparent elevation of cytochrome P-450 was obtained (Fig. 5, A and B), enabling observation of three forms of the hemoprotein by off-balance spectroscopy; that absorbing at 384 nm, the form with a peak at 420 nm and that with a peak at 454 nm. However, when higher amounts of SKF 525-A were administered, a very real induction of the P-450 hemoprotein (about 2-fold) was obtained. Treatment with SKF 525-A, although not always increasing the level of cytochrome P-450, did always elevate the rate of mixed function oxidation of aminopyrine and aniline (but the ratio of the two activities did not remain constant) without any apparent alteration of K_m values.

Anders *et al.*[15] reported that during metabolism of SKF 525-A two products were found. On the basis of gas–liquid chromatography and thin-layer chromatography, one of the products was identified as 2-ethylaminoethyl 2,2-diphenylvalerate (SKF 8742A, the *N*-monoethyl analogue of SKF 525-A); the other metabolite (called metabolite I) was not identified, but was shown not to be the primary amine (SKF 26754A). The fact that SKF 26754A also generated the 455 nm oxygenated complex of cytochrome P-450 (Fig. 4) suggests that this compound is also metabolized. The rate of formation of the 455 nm peak from SKF 26754A was about four times faster than from SKF 525-A. It may be that the latter compound is metabolized to the primary amine, which then is rapidly metabolized to some other product (perhaps metabolite I of Anders *et al.*[15]). When the metabolite is bound to the hemoprotein,

the latter is stabilized in a reduced form from which oxygen does not remove an electron (or a form which cannot obtain the necessary second electron to activate oxygen).

The species absorbing at 455 nm is present in the ferrous form, as indicated by its disappearance on addition of the oxidant, potassium ferricyanide. The peak also disappeared on addition of the chemical reductant, sodium dithionite, but reappeared after gassing the medium with oxygen; this latter effect was not obtained when oxygen was added after potassium ferricyanide. These observations indicated that the 455 nm species is the ferrous form of the hemoprotein in complex with oxygen and a metabolite. In the absence of metabolite the complex did not appear. Addition of sodium dithionite to liver microsomes, in the absence of SKF 525-A or SKF 26754A metabolism, followed by gassing with oxygen did not cause formation of a 455 nm peak.

In conclusion, the noncompetitive inhibition obtained after metabolism of SKF 525-A by the mixed function oxidase appears to be the result of the generation of a metabolite; this metabolite when combined with cytochrome P-450 stabilizes it (both *in vivo* and *in vitro*) in a reduced form capable of complexing with oxygen and giving rise to a 455 nm absorption peak. The inhibition is due to a failure to activate oxygen either because of the inability of the hemoprotein to receive a second electron or because of the inability to transfer the electron from the Fe^{2+} to oxygen.

Long-term administration of SKF 525-A to immature rats had no apparent effect on growth. Male rats, however, did show a slight increase in testicular weight, but this may be attributable to a slightly lower weight gain.

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